ABSTRACT  Bacteria that colonize the intestinal tract can invade epithelial cells or produce toxins that cause diarrhoeal diseases. Proliferation of *Clostridium perfringens* and production of alpha-toxin, a phospholipase C, is the major factor for necrotic enteritis in poultry. However, little is known about the functional importance of luminal alpha-toxin during intestinal infection. The purpose of this study was to investigate the effects of purified alpha toxin of *Clostridium perfringens* on the electrophysiology of the laying hen’s stripped jejenum in Ussing chambers. The effects were investigated in Experiment 1 after toxin addition to the mucosal and serosal side of the tissue, and a second experiment was performed to study the effect of the toxin on sodium-dependent glucose transport. Mucosal exposure of jejunal tissue sheets to 100 units of alpha toxin/L did not elicit electrophysiologic changes. The addition of purified alpha toxin to the serosal side induced a biphasic increase in short-circuit current ($I_{sc}$) after 15 and 100 min. The magnitude of the increase of $I_{sc}$ of both peaks was similar, but the second phase response lasted longer. The tissue conductivity tended ($P = 0.07$) to be lower after 2 h of toxin addition compared with basal value when no toxin was added. In the second experiment, adding D-glucose on the mucosal side of the jejenum increased ($P < 0.05$) the $I_{sc}$ from a baseline value of 42 ± 28 μA/cm² to a maximal value of 103 ± 27 μA/cm². Preincubation with α-toxin almost fully inhibited this stimulation of $I_{sc}$ by D-glucose. The conductance of the tissues was not affected by the toxin addition. These findings indicate that alpha toxin not only causes electrogenic secretion of anions, probably due to the stimulation of chloride secretion, but also diminishes electrogenic Na⁺/glucose cotransport from the mucosal to serosal side in the small intestine of poultry.

Key words: laying hen, *Clostridium perfringens*, alpha toxin, phospholipase C, Ussing chamber

INTRODUCTION

*Clostridium perfringens*, a widely distributed pathogen, is a low G + C gram-positive anaerobic spore-forming bacterium (Shimizu et al., 2002). This bacterium is ubiquitous in the environment and a member of the normal gut flora of animals (Collier et al., 2003). *Clostridium perfringens* produces different toxins (Smedley et al., 2004), and on the basis of the ability to produce major lethal toxins, the alpha, beta, epsilon, and iota, *C. perfringens* isolates are grouped into 5 toxin types (Type A–E; MacLennan, 1962; McDonel, 1980). The alpha toxin from *C. perfringens* was the first bacterial protein shown to possess enzymatic and toxic properties (MacFarlane and Knight, 1941). This toxin has a phospholipase C (PLC) activity and a calcium-dependent phospholipid binding domain (Titball et al., 2000). Phospholipases are a group of enzymes, and the role of these enzymes in the pathogenesis of infectious disease is diverse. A key event is the interaction of the enzyme with phospholipids in eukaryotic cell membranes (Titball, 1993). Alpha toxin exhibits haemolytic, necrotic, vascular permeabilizing, and platelet aggregating properties (Titball, 1993; Titball et al., 1999). It hydrolyzes the phosphatidylcholine and sphingomyelin moieties in the presence of calcium ions and promotes membrane disorganization (Titball, 1993; Naylor et al., 1998; Titball et al., 1999). Hydrolysis of lecithin results in a formation of diacylglycerol, resulting in the activation of protein kinase C and subsequent stimulation of the arachidonic acid cascade. This induces the synthesis of inflammatory mediators such as leukotriens, thromboxanes, platelet-agglutinating factors, and prostacyclins (Titball, 1993; Bunting et al., 1997; Titball et al., 1999). Analysis of the alpha toxin of chicken isolates of *C. perfringens* demonstrated high homology to mammal-derived strains (Sheedy et al., 2004). Conditions that promote the excessive growth of *C. perfringens* in chicken intestine lead to toxin production that causes necrotic enteritis (NE).

When alpha toxin was inoculated into ligated intestinal loops of adult pigs, it did not induce substantial lesions...
or fluid loss (Jestin et al., 1985). However, when administered intragastrically to neonatal piglets, alpha toxin causes edema of villi and neutrophilic inflammation of the small intestine (Johannsen et al., 1993a,b). Administration of semipurified ultrafiltered alpha toxin in ovine ileal and colonic loops in vivo induced accumulation of fluid in the lumen (Miyakawa and Uzal, 2005). Alpha toxin induced the contraction of isolated ileum (Sakurai et al., 1990) and neutrophilic enteritis when injected into small intestinal loops of rats (Otamiri, 1989). Serosal addition of 100 units of purified PLC to rat colon has been reported to induce a biphasic current because of chloride ion dependent secretion (Diener et al., 1991).

In poultry, the functional importance of intestinal alpha toxin release in the pathogenesis of NE is unclear. To our knowledge, no information is available regarding the changes in the electrophysiological parameters of the small intestine and glucose absorption induced by alpha toxin of *C. perfringens*. Therefore, the electrophysiological changes and the effects on electrogenic sodium glucose transport were studied in vitro in jejunal strips from laying hens in the Ussing chambers.

**MATERIALS AND METHODS**

**Birds, Feeding, and Housing**

Lohmann Brown laying hens (n = 13), 72 wk of age, were procured from a local commercial farm (Österr. Suppenhühner Verwertungs AG, Weistrach, Austria). The hens, weighing 1.5 to 2.0 kg, were housed on deep wood shavings litter and were fed a commercial diet (Tagger Feed Mill, Graz, Austria). The diet contained 17.0% crude protein, 5.0% crude fat, 4.3% crude fiber, 13.3% crude ash, and 0.4% methionine. The hens were provided diets and water ad libitum for the duration of the experiment. The actual experiment was started a week after the arrival of the birds in order to acclimatize them in a new environment.

**Tissue Preparation**

The hens were killed by stunning and bleeding at the Institute of Nutrition, University of Veterinary Medicine, Vienna, Austria. The jejunum was then removed directly after exsanguination, rinsed several times with ice-cold Ringer buffer (4°C), and transported in ice-cold oxygenated buffer to the laboratory within 5 min. The composition (mmol/L) of Ringer solution was CaCl₂, 1.2; MgCl₂, 1.2; Na₂HPO₄, 2.4; NaH₂PO₄, 0.4; NaHCO₃, 25; KCl, 5; NaCl, 15; and mannitol, 20; the pH was adjusted to 7.4. The segments taken from the proximal jejunum and tissue flaps were prepared by the method as described by Awad et al. (2005b). The intestinal segment was opened longitudinally along the mesenteric border and washed free of intestinal contents several times with Ringer solution at 4°C. Tissues were placed in the same cold Ringer buffer and gassed with carbogen (O₂/CO₂ ratio 95:5) until they were mounted in the Ussing chamber.

**Chemicals**

α-Toxin [phospholipase C (Sigma-Aldrich, Deisenhofen, Germany), EC 3.1.4.3, Type 1] from *C. perfringens* was dissolved in Ringer solution. The specific activity of PLC was 25 units/2.6 mg of solid. In each experiment, alpha toxin was reconstituted freshly. All the salts used to prepare the Ringer solution were of analytical grade and were purchased from Sigma-Aldrich.

**Measurements of Electrophysiological Traits**

Short-circuit current (I₇c), tissue conductance, and transmural potential difference were measured in the Ussing chamber with a microprocessor system based on a voltage/current clamp device (Mussler, Microclamp, Aachen, Germany).

The serosa and muscularis were stripped manually to obtain a mucosa-submucosa preparation of the jejunum. Thereafter, the epithelial sheets were mounted in the modified Ussing chambers with an exposed tissue area of 1 cm². The serosal and mucosal surfaces of the tissues were bathed in 5 mL of Ringer solution with the same composition described previously. The bathing medium in the chambers was aerated with 95% O₂ and 5% CO₂ and maintained at 38°C in a water bath (Julabo Inc., Allentown, PA). The solution was continually stirred and oxygenated by bubbling into the chamber by means of a gas lift. The electrode potential and the solution resistance were determined at the beginning of every experiment and were automatically corrected before tissues were placed in the chamber. The tissues were first incubated under open circuit conditions for 30 min for equilibration and were short-circuited after that by clamping the voltage at 0 mV. The toxin was added when the base line was achieved.

**Experimental Design**

**Experiment 1.** This experiment was performed to determine whether alpha toxin stimulates ionic secretion (n = 6). The toxin was added to the mucosal compartment of the Ussing chamber where no response of the toxin was noted on the short-circuit current. Further experiments were performed to investigate the effects of toxin after addition to the serosal compartment of the Ussing chamber. The final concentration of the toxin was 100 U/L of Ringer solution.

**Experiment 2.** The second experiment was carried out to investigate the effect of alpha toxin on sodium-dependent glucose absorption (n = 7). The proximal jejunum sheets were preincubated with the toxin (100 U/L) for 30 min. Thereafter, tissues were short-circuited unless a stable base line was achieved. Later, the Ringer solution in the mucosal side was replaced by the same solution with 5 mmol/L of D-glucose instead of mannitol. The electrical response was measured as the peak response obtained 1 min after the addition of glucose. Six jejunal
strips were prepared from individual birds in each set of experiment.

**Statistics**

Statistical program SPSS (version 11.5, SPSS GmbH, SPSS Inc., Munich, Germany) was used for data analysis. The Kolmogorov Smirnov test was used to test the normal distribution of the data. Results are expressed as means ± SEM. Repeated measures ANOVA procedure was used to investigate the effect of alpha- and D-glucose on the Isc, tissue conductance, and transmural potential difference (3 replicates/bird) and subsequent paired t-test. In Experiment 2, independent sample t-test was used for comparison between control and toxin group. Probability values of less than 0.05 (P < 0.05) were considered significant.

**RESULTS**

**Experiment 1**

After an equilibration time of 30 min, the tissues were short-circuited until a stable basal line was achieved prior to the addition of alpha toxin. No change in the Isc was observed when the toxin was added to the mucosal compartment of the Ussing chamber. Even the addition of a higher concentration of alpha toxin (10³ U/L) failed to elicit any change in Isc when added to the mucosal side (data not shown). However, administration of alpha toxin in a concentration of 100 U/L to the serosal side induced a biphasic increase in Isc in the jejunal strips. The first increase (44 ± 13 µA/cm²) was observed after 15 min, which was higher (P < 0.001) than the basal Isc (22 ± 12 µA/cm²). Afterwards, the Isc began to fall and approached to the baseline (P > 0.05) within 40 min (10 ± 10 µA/cm²). Then, it started to rise again in the second phase, and maximum response of alpha toxin in terms of Isc (41 ± 11 µA/cm²) was observed after 100 min which was higher (P < 0.05) than the base-line (22 ± 12 µA/cm²). The second phase of Isc response was statistically not different in magnitude from the first phase (P > 0.05); however, the response of the second phase remained for a longer time, and it reached the basal value after 200 min (±5 ±13 µA/cm², P > 0.05). The tissue conductance (mS/cm²) tended to be lower (P = 0.07) 2 h postaddition of alpha toxin (9 ± 5) compared with the basal value before the administration of alpha toxin (11 ± 5).

**Experiment 2**

Addition of D-glucose to the mucosal compartment resulted in an increase of Isc in treated and control tissues (Table 1) when compared with their respective basal values. In control tissues that were not preincubated with toxin, the increase of Isc (ΔIsc) was 60.6 µA/cm², whereas the tissues incubated with alpha toxin had a diminished response of 13.4 µA/cm² compared with the baseline Isc (P < 0.05). The addition of D-glucose decreased the transmural potential difference in the control group as well as in the jejunal sheets incubated with toxin; there was a greater decrease (P = 0.059) in the control group compared with the treatment group (Table 2). The tissue conductivity was not affected at any time interval when control and toxin-treated groups were compared (Table 3).

**DISCUSSION**

In chickens, the toxin of *C. perfringens* has been implicated in the pathogenesis of NE with diarrhoea and macroscopic lesions in the small intestine. The incidence of NE in poultry has increased in many countries, probably due to the reduced utilization of antibiotic growth promoters (Immerseel et al., 2004).

To our knowledge, no study has been carried out to investigate in vitro the effect of alpha toxin of *C. perfringens* on the sodium, chloride, or glucose transport in chicken intestine, and only scanty data are available in other species (Diener et al., 1991; Miyakawa and Uzal, 2005). The experiment described in the present report demonstrated an effect of purified alpha toxin on the electrophysiology of the intestine of laying hens. Addition of alpha toxin to the luminal compartment of the Ussing chamber did not induce changes in the Isc. The results are in agreement with studies in rats (Diener et al., 1991), which also failed to observe changes in the Isc when alpha toxin was added to the mucosal side of rat colon. It may

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**Table 1.** Short-circuit current (µA/cm²) of the isolated jejunal tissues from laying hens with or without preincubation with alpha toxin

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Toxin preincubated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>42 ± 28</td>
<td>31 ± 11</td>
<td>0.62</td>
</tr>
<tr>
<td>After glucose addition</td>
<td>103 ± 27</td>
<td>44 ± 13</td>
<td>0.03</td>
</tr>
<tr>
<td>30 minutes post glucose addition</td>
<td>23 ± 30</td>
<td>16 ± 12</td>
<td>0.80</td>
</tr>
</tbody>
</table>

1Values are expressed as means ± SEM of 7 birds (3 replicates/bird).

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**Table 2.** Transmural potential difference (mV) across the isolated jejunal tissues from laying hens with or without preincubation with alpha toxin

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Toxin preincubated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>-3 ± 2</td>
<td>-2 ± 1</td>
<td>0.51</td>
</tr>
<tr>
<td>After glucose addition</td>
<td>-7 ± 2</td>
<td>-3 ± 1</td>
<td>0.059</td>
</tr>
<tr>
<td>30 min post glucose addition</td>
<td>-2 ± 2</td>
<td>-2 ± 1</td>
<td>0.78</td>
</tr>
</tbody>
</table>

1Values are expressed as means ± SEM of 7 birds (3 replicates/bird).

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**Table 3.** Tissue conductance (mS/cm²) of the isolated jejunal tissues from laying hens with or without preincubation with alpha toxin

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Toxin preincubated</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>13 ± 2</td>
<td>19 ± 3</td>
<td>0.25</td>
</tr>
<tr>
<td>After glucose addition</td>
<td>12 ± 1</td>
<td>16 ± 2</td>
<td>0.29</td>
</tr>
<tr>
<td>30 min post glucose addition</td>
<td>14 ± 6</td>
<td>19 ± 2</td>
<td>0.29</td>
</tr>
</tbody>
</table>

1Values are expressed as means ± SEM of 7 birds (3 replicates/bird).
be speculated that additional co-factor is required for this toxin to elicit its action on mucosal membrane of the gastrointestinal tract. This toxin may bind with mucus, thus hampering its diffusion to the apical membrane of the enterocytes. *Clostridium perfringens* is also able to grow on medium with mucin as a substrate, indicating that this organism possesses mucolytic activity (Deplancke et al., 2002). The genome sequence of *C. perfringens* showed that this bacterium also secretes exo-alpha sialidase (Shimizu et al., 2002). Mucolysis could serve as an initiating step of *C. perfringens* virulence and point to a possible link between bacterial mucolysis in the chick’s intestine and intestinal barrier function (Collier et al., 2003).

Alpha toxin induced a biphasic increase in $I_{sc}$ when the toxin was added to the serosal compartment of the Ussing chamber. The first peak was attained after 15 min, and the second peak was reached after about 100 min. The results are similar to the findings of Diener et al. (1991) who also observed 2 peaks when rat colon was exposed to the alpha toxin on the serosal surface. The second peak, in the present study, did not differ from the first peak ($P > 0.05$ in terms of magnitude of $I_{sc}$; however, Diener et al. (1991) demonstrated a higher increase in $\Delta I_{sc}$ in the second peak as compared with the first peak. It is possible that alpha toxin from different isolates of *C. perfringens* toxotype or even from different isolates of *C. perfringens* type A have different morphologic or physiological effects on the intestine (Miyakawa and Uzal., 2005).

The ionic mechanism of the increase in $I_{sc}$ in the first peak may be due to the electrogenic secretion of chloride anions. The first and immediate response may be due to the release of arachidonic acid, probably from the connective tissue of the submucosa and lamina propria. The toxin first encounters the connective tissue of submucosa and lamina propria before penetrating to the enterocytes. The second phase of alpha toxin response may be due to the release of arachidonic acid involving protein kinase C (Diener et al., 1991).

In the intestine, D-glucose and amino acids are absorbed by 2 different mechanisms: paracellular, passive diffusion and transcellular transport (Garcia-Amado et al., 2005). In the former, no energy is required, whereas in transcellular transport, there is an active, energetically costly absorption of D-glucose and amino acids (Pappenheimer, 1993). The selection of jejunum as an experimental tissue depends upon the fact that it is the most efficient segment for Na-mediated uptake of glucose through the SGLT1 carrier protein (Amat et al., 1996).

In birds, the small intestine and certain regions of large intestine such as the colon transport glucose with Na+ (Lind et al., 1980; Ferrer et al., 1986; Moreto and Planas, 1989; Bindslev et al., 1997; Awad et al., 2005a). Glucose and other nutrients are absorbed from the intestinal lumen by active carrier transport, often coupled with Na+. If glucose is added to the mucosal side of the intestinal tissues, a carrier-mediated transport is stimulated, with a rise in the uptake of Na+. The brush border membrane depolarization and the rise in cytosolic sodium concentration stimulate Na+-K+ ATPase, which, in turn, increases the net flux of sodium from the luminal to the serosal side. All of these events modify the electrical parameters of the intestinal tissue and increase $I_{sc}$ (Shimada and Hoshi, 1986; Wright et al., 1994; Amat et al., 1999). In the present study, the addition of 5 mmol/L of D-glucose to the mucosal side caused a significant increase in $I_{sc}$ in control tissues and the tissues preincubated with alpha toxin (Table 1), which is due to the stimulation of transepithelial Na+ flux. The response to mucosal addition in toxin-exposed jejunum was decreased ($P < 0.05$) in comparison with the response of the control tissue. The decreased response to glucose could indicate that diarrhoea caused by the alpha toxin is not only secretory and chloride mediated but also caused by sodium malabsorption or, at least, by a substantially diminished substrate-dependent Na+-absorption from the jejunal epithelial Na+ flux. The toxin may increase the production of mucus that might have caused an increase in the thickness of the unstirred layer surrounding the enterocytes, thus impeding the diffusion of glucose toward the apical membrane of the enterocytes.

The tissue conductance was decreased ($P = 0.07$) 2 h postaddition of alpha toxin. This may be due to an increase in paracellular or transcellular resistance or both. On the other hand, Otamiri (1989) suggested that this toxin might impair the function of the mucosal barrier and increase the permeability of the intestine. Therefore, this aspect needs further experimentation involving the role of various proteins of the tight junction of enterocytes in the mediation of regulation of barrier function of the gastrointestinal tract following alpha toxin treatment.

In conclusion, the serosal addition of alpha toxin caused a biphasic increase in $I_{sc}$ in jejunum sheets from laying hens. Both peaks were similar in magnitude, but the second peak remained for a longer time. This toxin also caused a decrease in sodium-dependent glucose absorption from the gut mucosa. These findings may be helpful in understanding the pathogenicity of alpha toxin.

**REFERENCES**


